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<p>(54) Title: A COMPOSITION CONTAINING A TETRACYCLINE AND USE FOR INHIBITING ANGIOGENESIS</p> <p>(57) Abstract</p> <p>Pharmaceutical compositions for delivering an effective dose of an angiogenesis inhibitor consisting of a tetracycline such as a minocycline or chemically modified tetracycline retaining collagenase inhibitory activity but not antibacterial activity. The effective dosage for inhibition of angiogenesis based on <i>in vitro</i> testing is between one and 500 micromolar. The compositions are delivered topically, locally or systemically using implants or injection, alone or in combination with a chemotherapeutic agent such as <math>\beta</math>-cyclodextrin. The composition is extremely selective for growth of endothelial cells, inhibiting growth, but is not cyto- toxic at the effective dosages.</p>		

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A COMPOSITION CONTAINING A TETRACYCLINE AND USE FOR  
INHIBITING ANGIOGENESIS

Background of the Invention

This invention is in the field of angiogenesis inhibitors, in particular antibiotics that inhibit angiogenesis.

The U.S. government has rights in this invention by virtue of a grant from the National Institutes of Health, grant number  
5 NSO1058-01.

Angiogenesis, the proliferation and migration of endothelial cells that result in the formation of new blood vessels, is an essential event in a wide variety of normal and pathological processes. For example, angiogenesis plays a critical role in embryogenesis, wound  
10 healing, psoriasis, diabetic retinopathy, and tumor formation, as reported by Folkman, J. *Angiogenesis and its inhibitors*. In: V. T. DeVita, S. Hellman and S. A. Rosenberg (eds.). Important Advances in Oncology, pp. 42-62, (J. B. Lippincott Co., Philadelphia, 1985); Brem, H., et al., *Brain tumor angiogenesis*. In: P. L. Kornblith and  
15 M. D. Walker (eds.), Advances in Neuro-Oncology, pp. 89-101. (Future Publishing Co., Mount Kisco, NY 1988); Folkman, J. *Tumor angiogenesis: therapeutic implications*. N. Engl. J. Med., 285: 1182-1186 (1971); and Folkman, J. *Successful treatment of an angiogenic disease*. N. Engl. J. Med., 320: 1211-1212 (1989).

20 Identification of several agents that inhibit tumor angiogenesis has provided a conceptual framework for the understanding of angiogenesis in general. The inhibition of angiogenesis by certain steroids and heparin derivatives, reported by Folkman, J., et al., Science 221: 719 (1983); and Murray, J. B., et  
25 al., *Purification and partial amino acid sequence of a bovine cartilage-derived collagenase inhibitor*. J. Biol. Chem., 261: 4154-4159 (1986); led to studies elucidating the crucial role of remodeling of the

-2-

extracellular matrix in angiogenesis. These agents apparently prevent angiogenesis by specifically disrupting the deposition and cross-linking of collagen, as reported by Ingber, D., and Folkman, J. *Inhibition of angiogenesis through modulation of collagen metabolism.* Lab.

5 Invest., 59: 44-51 (1989).

The original description of angiogenesis inhibition in the presence of cartilage, reported by Brem, H., et al., J. Exp. Med. 141: 427-439 (1975); Brem, H., et al., Extracellular Matrices Influences on Gene Expression pp. 767-772 (Academic Press, NY 1975); and

10 Langer, R., et al., Science 70-72 (1976); led to the isolation and purification from bovine cartilage of a protein fraction that not only inhibited angiogenesis but inhibited protease activity, described by Murray, J.B., et al., J. Biol. Chem. 261: 4154-4159 (1986).

Subsequently, an extract derived from the vitreous of rabbits was  
15 shown to inhibit tumor angiogenesis by Brem, S., et al., Am. J. Ophthalm. 84: 323-328 (1977). The demonstration that heparin alone enhanced the angiogenesis response buttressed the hypothesis that heparin produced by mast cells that had migrated to the tumor site facilitated the development of new capillaries, as reported by Kessler,  
20 D.A., et al., Int. J. Cancer 18:703-709 (1976).

Recent studies on inhibition of angiogenesis have highlighted the importance of enzyme mediated remodeling of the extracellular matrix in capillary growth and proliferation (Folkman, J., et al., Science 221: 719-725 (1983); Ingber, D., et al. Lab. Invest.  
25 59: 44-51 (1989); Folkman, J., et al., Science 243: 1490-1493 (1989); Krum, R., et al., Science 230: 1375-1378 (1985); Ingber, D., et al., Endocrinol. 119: 1768-1775 (1986); and Ingber, D., et al., J. Cell Biol. 109: 317-330 (1989)). It has been suggested by Ingber, D., et al., Lab. Invest. (1989) and Endocrinol. (1986) that the steroid-heparin  
30 combination is involved in the dissolution of the capillary basement membrane by inhibiting the deposition or cross-linking of collagen.

SUBSTITUTE SHEET

-3-

The isolation of a collagenase inhibitor from cartilage, the first source of an angiogenesis inhibitor, was of interest since it suggested that angiogenesis may be inhibited not only by disrupting collagen deposition, but also by interrupting collagen breakdown. It is therefore possible that a combination of agents that interfere with both the anabolic and catabolic phases of collagen metabolism will prove even more effective in halting tumor angiogenesis.

A number of investigators have reported that extracts of cartilage, one of the few avascular tissues in the body, can inhibit angiogenesis: Eisentein, et al., Am. J. Pathol. 81, 1-9 (1987); Pauli, et al., J. Natl. Cancer Inst. 67,55-74 (1981); Brem and Folkman, J. Exp. Med. 141, 427-439 (1975); Langer, et al., Science 193, 70-72 (1976); Langer, et al., Proc. Natl. Acad. Sci. USA 77, 431-435 (1980); and Lee and Langer, Science 221, 1185-1187 (1983). Langer, et al., showed that cartilage extracts containing a collagenase inhibitor retard tumor-induced and inflammatory-induced neovascularization in the cornea and conjunctiva, when delivered by either infusion or sustained release from a polymeric implant.

The potential therapeutic benefit that an effective, economic, well characterized inhibitor of angiogenesis might have in controlling diseases in which neovascularization plays a critical role has prompted a long term search for angiogenesis inhibitors. There are many advantages to having such an inhibitor that can be prepared by simple organic synthesis, rather than by cloning and expression of a protein, or synthesis of a long polypeptide. An inhibitor of angiogenesis could have an important therapeutic role in relieving the course of these disorders, as well as provide a valuable means of studying their etiology.

It is therefore an object of the present invention to provide a pharmaceutical composition, and method of use thereof, for the treatment of diseases involving abnormal angiogenesis.

-4-

It is another object of the present invention to provide topical and controlled release pharmaceutical compositions, and methods of use thereof, for inhibition of angiogenesis.

It is still another object of the present invention to provide  
5 an economical, well characterized, composition for inhibition of angiogenesis.

### Summary of the Invention

An antibiotic composition that is an effective inhibitor of angiogenesis has been developed. The preferred antibiotic is  
10 minocycline, although other tetracycline-like antibiotics that inhibit collagenase are also effective. Inhibition of endothelial proliferation by these compounds is associated with collagenase inhibition, does not require antibiotic activity, and is unrelated to cytotoxicity.

The effective dosage for inhibition of angiogenesis *in vivo*  
15 is extrapolated from *in vitro* and *in vivo* inhibition assays. Effective dosages range from approximately one micromolar to 500 micromolar. The dosage range is much higher than the dosage used for inhibition of bacterial growth. The effective dosage is somewhat dependent on the method and means of delivery. For example, in some  
20 applications, as in the treatment of psoriasis or diabetic retinopathy, the inhibitor is delivered in a topical carrier. In other applications, as in the treatment of solid tumors, the inhibitor can be delivered by means of a biocompatible, biodegradable or non-degradable, polymeric implant, systemically, or by local infusion.

25 Effectiveness was demonstrated by incorporating minocycline into controlled release polymers and testing in the rabbit cornea against neovascularization in the presence of the VX2 carcinoma. Inhibition by minocycline was shown to be comparable to that of the combination of heparin and cortisone, a potent inhibitor of

-5-

angiogenesis. Minocycline decreased tumor-induced angiogenesis by a factor of 4.5, 4.4 and 2.9 at 7, 14 and 21 days, respectively. At the end of the study, none of the corneas with minocycline had such vascular masses, in contrast to the corneas with empty polymers, which had large, invasive, exophytic tumors. Further studies demonstrate the selectivity of the compound for endothelial cells.

### Brief Description of the Drawings

Figure 1 is a graph comparing the extent of inhibition of angiogenesis (Angiogenesis index) in rabbit cornea over time (days) for the control (squares); cortisone (circles); heparin in combination with cortisone (triangles); and minocycline (+).

Figure 2A, 2B, and 2C are graphs demonstrating the selective inhibition of endothelial cell growth by minocycline: Figure 2A graphs cells (% of control) for three cell types: pericytes (empty squares), astracytes (dark circles), and endothelial cells (dark squares) versus concentration of minocycline (micromolar). Figure 2B graphs DNA synthesis (cpm  $^3\text{H}$ -thymidine/microgram protein, % of control) versus concentration of minocycline (micromolar). Figure 2CB graphs doubling time (% control) for paraaortic endothelial cells (empty squares) and bovine retinal endothelial cells (dark diamonds) versus concentration of minocycline (micromolar).

Figures 3A and 3B are graphs demonstrating the inhibition of endothelial cell proliferation by minocycline,  $\beta$ -cyclodextrin (B-CD), and combinations thereof: Figure 3A graphs cells (% of control) versus concentration (1.0  $\mu\text{M}$  to 10  $\mu\text{M}$ ) for minocycline alone (open box), B-CD alone (dark triangle), 0.5:1 B-CD:Min (dark square with light dot), 1:1 B-CD:Min (open triangle), 2:1 B-CD:Min (dark square) and Figure 3B graphs % control minocycline alone (open triangle), B-CD alone (dark square), 0.5:1 B-CD:Min (open square with dark dot).

**SUBSTITUTE SHEET**

-6-

1:1 B-CD:Min (dark triangle), 2:1 B-CD:Min (dark square with light dot).

### Detailed Description of the Invention

Antibiotics effective as inhibitors of angiogenesis have been  
5 discovered. The antibiotics are tetracyclines inhibiting collagenase.  
The preferred antibiotic is minocycline, a semisynthetic tetracycline  
antimicrobial with anticollagenase properties.

#### Angiogenesis Inhibitors

The antibiotics that are useful as angiogenesis inhibitors are  
10 those having collagenase inhibitory activity. These include the  
tetracyclines and chemically modified tetracyclines (CMTs), and three  
ringed tetracycline homologs, that have the ability to inhibit  
collagenase but diminished antibacterial activity.

The tetracyclines are characterized by four carbocyclic  
15 rings. Most are effective against a broad range of pathogens, as  
reported by Laskin, A. J. *Tetracyclines*, In: D. Gotlieb and P. D.  
Shaw (eds.), *Antibiotics*, pp. 331-359 (Springer-Verlag, NY 1967).  
They bind to the bacterial 30S ribosome, block access of the  
aminoacyl tRNA to the binding site on the mRNA-ribosome complex,  
20 and thereby inhibit protein synthesis by preventing the addition of  
amino acids to the growing peptide chain. The tetracyclines usually  
spare protein synthesis in mammalian cells since these cells lack the  
active transport system found in bacteria.

Examples of commercially available tetracyclines include  
25 chlortetracycline, demeclocycline, doxycycline, lymecycline,  
methacycline, minocycline, oxytetracycline, rolitetracycline, and  
tetracycline. The active salts, which are formed through protonation  
of the dimethylamino group on carbon atom 4, exist as crystalline

-7-

compounds. These are stabilized in aqueous solution by addition of acid.

Minocycline, a semisynthetic tetracycline antimicrobial, described by Martell, M. J., and Boothe, J. H. in J. Med. Chem., 10: 44-46 (1967), and Zbinovsky, Y., and Chrikian, G. P. Minocycline. In: K. Florey (ed.), Analytical Profiles of Drug Substances, pp. 323-339 (Academic Press, NY 1977), the teachings of which are incorporated herein, has anticollagenase properties, as reported by Golub, L. M., et al., , J. Periodontal Res., 18: 516-526 (1983); Golub, L. M., et al., J. Periodontal Res. 19: 651-655 (1984); Golub, L. M., et al., J. Periodontal Res. 20: 12-23 (1985); and Golub, L. M., et al., J. Dent. Res., 66: 1310-1314 (1987).

Minocycline, first described in 1967, is derived from the naturally produced parent compounds chlortetracycline and oxytetracycline.

Minocycline inhibits collagenase activity directly by a mechanism unrelated to its antimicrobial properties. Minocycline has been shown to inhibit collagenolysis and cytolysis induced by melanoma-produced metalloproteases *in vitro*, as reported by Zuker, S., J. Natl. Cancer Inst. 75:517-525 (1985), and the collagenase activity in the synovial fluid of patients with rheumatoid arthritis, as reported by Greenwald, R.A., et al., J. Rheumatol. 14:28-32 (1987). The mode of action of minocycline on collagenase is not well understood.

The chemically modified tetracyclines are described by U.S. Patent No. 4,704,383 to McNamara, et al., 4,925,833 to McNamara, et al., 4,935,411 to McNamara, et al., the teachings of which are incorporated herein. As used herein, these are tetracyclines having substantially no effective antibiotic activity but having collagenase inhibitory activity. An example is dedimethylaminotetracycline. Other compounds include 7-chlorotetracycline, 5-hydroxytetracycline, 6-demethyl-7-

-8-

chlorotetracycline, 6-demethyl-6-deoxy-5-hydroxy-6-methylenetetracycline, 6-alpha-benzylthiomethylenetetracycline, a nitrile analog of tetracycline, a mono-N-alkylated amide of tetracycline, 6-fluorodemethyltetracycline, 11-alpha-chlorotetracycline, 2-acetyl-8-hydroxy-1-tetracycline and 6-demethyl-6-deoxytetracycline.

A number of tetracycline analogs can be synthesized with side-chain deletions or, in some cases, moieties added to the parent tetracycline molecule. One category, described by McCormick, et al., "Studies of the reversible epimerization occurring in the tetracycline family. The preparation, properties, and proof of structure of some 4-epi-tetracyclines", J. Am. Chem. Soc. 79, 2849 (1957); Boothe, et al., Chemistry of the tetracycline antibiotics. I. Quaternary derivatives, J. Am. Chem. Soc. 80, 1654 (1958); and McNamara, et al., "The synthesis and characterization of a non-antibacterial chemically-modified tetracycline (CMT)", J. Dent. Res. 65 (Spec. Issue), IADR abstr. no. 515 (1986), the teachings of which are incorporated herein. This alteration involved the removal of the dimethylamino group from the carbon-4 position of the "A" ring, resulting in the CMT called 4-de-dimethylaminotetracycline. This compound has collagenase inhibitory activity but not antimicrobial activity. Other examples are tetracyclinonitrile (produced by dehydration of the carboxamide residue at carbon 2 of the tetracycline molecule), 6-deoxy 6-demethyl 4-de-dimethylaminotetracycline (produced by removing the hydroxyl and methyl groups on carbon 6 from 4-de-dimethylaminotetracycline), and 7-chloro 4-de-dimethylaminotetracycline (produced by removing the dimethylamino group from carbon 4 of chlortetracycline). Based on studies conducted by Golub, et al., "Tetracyclines Inhibit Connective Tissue Breakdown: New Therapeutic Implications for an Old Family of Drugs", Critical Reviews in Oral Biology & Medicine, it is believed that the carboxamide residue at carbon 2 and the methyl and hydroxyl groups at carbon 6, as well as the dimethylamino group

at carbon 4, are not required to retain the anti-collagenase activity of the tetracycline molecule. However, it is believed that the carbonyl oxygen at carbon-11 and the hydroxyl group at carbon-12 are required for collagenase inhibitory activity.

5           The tetracyclines are ideally suited for inhibition of angiogenesis since they are readily available commercially, are innocuous to mammalian cells, and have been in clinical use for several years. One of the advantages of anti-collagenase agents as antineoplastic agents is that they can be used to inhibit pathological  
10 collagenolytic processes, while leaving physiologically necessary proteolytic processes unaffected. Specific collagenase inhibitors with low systemic toxicity can be very useful as modulators of tumor growth.

**Methods and compositions for administration to patients.**

15           The effective dosage range of minocyclin is between one and 500 micromolar, based on *in vitro* studies. The dosage optimum is 50 micromolar. No cytotoxicity is observed within this dosage range. The effective dosage range is much higher than the dosage range used for inhibition of bacteria, which is in the range of less than  
20 one micromolar. The effective dosage for the related compounds is similar and can be extrapolated from the *in vitro* data.

          The tetracyclines can be administered alone or in combination with, either before, simultaneously, or subsequently to treatment using conventional chemotherapeutic or radiation therapy. A  
25 preferred embodiment is the systemic administration, either by injection or implantation of polymeric encapsulated drug, of minocycline in combination with a chemotherapeutic such as  $\beta$ -cyclodextrin, in a ratio of between 0.5 and 2.0 to 1,  $\beta$ -cyclodextrin to minocycline. Other chemotherapeutics include carmustine (BCNU), 5-  
30 fluorouracil,  $\beta$ -cyclodextrin, vinca alkaloids such as vincristin, taxol and vinblastin, chlorambucil, cytoxan, alcoran, busulfan, methotrexate,

-10-

mercaptapurine, bleomycin, adriamycin, thioguanine, chlorotrianisene, cyclophosphamide, and derivatives of cyclophosphamide of 4-HC, immunotoxins.

Pharmaceutical compositions are prepared using the  
5 antibiotic as the active agent to inhibit angiogenesis based on the specific application. Application is either topical, localized, or systemic. Any of these compositions may also include preservatives, antioxidants, antibiotics, immunosuppressants, and other biologically or pharmaceutically effective agents which do not exert a detrimental  
10 effect on the antibiotic or cells. For treatment of tumors, the composition may include a cytotoxic agent which selectively kills the faster replicating tumor cells, many of which are known and clinically in use.

For topical application, the antibiotic is combined with a  
15 carrier so that an effective dosage is delivered, based on the desired activity, at the site of application. The topical composition can be applied to the skin for treatment of diseases such as psoriasis. The carrier may be in the form of an ointment, cream, gel, paste, foam, aerosol, suppository, pad or gelled stick. A topical composition for  
20 treatment of eye disorders consists of an effective amount of antibiotic in a ophthalmically acceptable excipient such as buffered saline, mineral oil, vegetable oils such as corn or arachis oil, petroleum jelly, Miglyol 182, alcohol solutions, or liposomes or liposome-like products.

25 Compositions for local or systemic administration, for example, into a tumor, will generally include an inert diluent. Solutions or suspensions used for parenteral, intradermal, subcutaneous, or topical application can include the following components: a sterile diluent such as water for injection, saline  
30 solution, fixed oils, polyethylene glycols; glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol

-11-

or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The  
5 parental preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

For directed internal topical applications, for example for treatment of hemorrhoids, the composition may be in the form of tablets or capsules, which can contain any of the following ingredients,  
10 or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; or a glidant such as colloidal silicon dioxide. When the dosage unit form  
15 is a capsule, it can contain, in addition to material of the above type, a liquid carrier such as a fatty oil. In addition, dosage unit forms can contain various other materials which modify the physical form of the dosage unit, for example, coatings of sugar, shellac, or other enteric agents.

20 In a preferred form, the composition is administered in combination with a biocompatible polymeric implant which releases the antibiotic over a controlled period of time at a selected site. Examples of preferred biodegradable polymeric materials include polyanhydrides, polyorthoesters, polyglycolic acid, polylactic acid,  
25 polyethylene vinyl acetate, and copolymers and blends thereof. Examples of preferred non-biodegradable polymeric materials include ethylene vinyl acetate copolymers.

The composition can be administered systemically using any of several routes, including intravenous, intracranial,  
30 subcutaneous, orally, or by means of a depo. The composition can be administered by means of an infusion pump, for example, of the type

SUBSTITUTE SHEET

-12-

used for delivering insulin or chemotherapy to specific organs or tumors, or by injection.

**Example 1: Demonstration of Effectiveness of minocycline in inhibiting angiogenesis.**

5 To test for inhibition of angiogenesis, compounds were impregnated into controlled release polymers, and assessed in the rabbit cornea, which allows direct, quantitative observation of neovascularization, as follows.

Animals. New Zealand White rabbits weighing about 1.5-  
10 2.5 kg were obtained from Bunnyville Farm (Littlestown, PA), kept in standard animal facilities, one animal per cage, and given free access to food and water.

Anesthesia. For the corneal implantations, subsequent stereomicroscopic examinations, and serial transplantation of the VX2  
15 tumor in the thigh, the animals were anesthetized with a mixture of xylazine, 15-17 mg/kg, and ketamine, 15-17 mg/kg, injected i.m.

VX2 Rabbit Carcinoma. The VX2 carcinoma, described by Kidd, J. G., and Rous, P.A. J. Exp. Med. 71: 813-838 (1940), a serially transplantable tumor syngeneic to the New Zealand White  
20 rabbit, was propagated by serial transplantation in the flank of New Zealand White rabbits.

Test Substances and Polymer Preparation. Ethylene-vinyl acetate copolymer (40% vinyl acetate by weight, Elvax 40P) was obtained from the DuPont Co., Wilmington, DE, as used as described  
25 by Langer, R., and Folkman, J. Nature (Lond.) 263: 797-800 (1976). The polymer was washed extensively in absolute ethyl alcohol, with total volume changes every 24 hours, to extract the inflammatory antioxidant butylhydroxytoluene. The presence of butylhydroxytoluene in the wash was monitored spectrophotometrically at 230 nm, and the  
30 washes were continued until the absorbance fell below 0.03 unit. The polymers were then dried in a vacuum desiccator for 5 days.

SUBSTITUTE SHEET

-13-

The agents to be tested for angiogenesis inhibition were incorporated into the ethylene-vinyl acetate copolymer (EVAc) matrix by modification of the fabrication procedure described by Rhine, W. D., et al., J. Pharm. Sci., 69: 265-270 (1980). Minocycline and

5 heparin crystals were ground to a fine powder and sieved through a 200 mesh (74  $\mu$ m) screen in a Collector tissue sieve (Belleo Glass, Inc., Vineland, NJ), to obtain a uniform sample consisting of particles less than 75  $\mu$ m in diameter. The final concentrations (w/w) of the angiogenesis inhibitors in the polymers were: (a) minocycline

10 hydrochloride (Sigma Chemical Co., St. Louis, MO), 10 and 20%; (b) cortisone acetate (Sigma Chemical Co.), 7.5 and 27%; (c) and heparin (Hepar Inc., Franklin, OH) and cortisone acetate, 15 and 30% combined loading, with a fixed 1:8 heparin:cortisone ratio. No significant differences were observed in the degree of angiogenesis

15 inhibition between the two loading levels for each drug.

Rabbit Cornea Angiogenesis Assay. The inhibition of angiogenesis was determined by assaying the degree of angiogenesis in the rabbit cornea in the presence of specified inhibitors, using the method described by Gimbrone, M. A., Cotran, R. S., Leapman, S.,

20 and Folkman, J. J. Natl. Cancer Inst. 32:413-427 (1974). The cornea provides an avascular matrix into which blood vessels grow and can be quantitated. A total of 116 corneas were implanted as follows: 50 corneas with VX2 carcinoma and empty polymer; 16 with VX2 carcinoma and minocycline polymer; 14 with VX2 carcinoma and

25 heparin/cortisone polymers; and 36 with VX2 carcinoma and cortisone polymers. Five corneas were excluded from the study; three became infected, one lost its polymeric implant, and one was lost when the rabbit died prior to the first reading on day 7.

The corneas were examined with a Zeiss slit lamp

30 stereomicroscope (Carl Zeiss, Inc., Thornwood, NY) on days 7, 14 and 21 after implantation. A total of 111 corneas were evaluated on

SUBSTITUTE SHEET

-14-

days 7 and 14, and 77 corneas were assessed on day 21. The angiogenesis response was quantitated by measuring both vessel length and vessel number to provide an angiogenesis index. For vessel length, the span of the blood vessels from the corneo-scleral junction to the leading edge of the new blood vessel front was measured with an ocular microscale eyepiece. The number of blood vessels present was designated, based on the following 4-level scale; 0, 0 vessels; 1, 1-10 vessels; 2, greater than 10 vessels, loosely packed so that details of the iris could be observed through the gaps between the vessels; and 3, greater than 10 vessels, tightly packed so that the iris could not be seen through the gaps between the vessels. An angiogenesis index was then determined as follows:

$$\text{angiogenesis index} = \text{vessel length} \times \text{vessel density}$$

**Histological Examination of Cornea.** The rabbits were sacrificed after the last stereomicroscopic examination on day 21 by the i.v. administration of T-61 Euthanasia Solution (Taylor Pharmacal Co., Decatur, IL). Representative corneas were removed and placed in phosphate-buffered formalin for 10-14 days, embedded in paraffin, sectioned with a microtome, and stained with hematoxylin and eosin for histological examination.

**Statistical Analysis.** The angiogenesis indexes for the four groups were compared by using the Kruskai-Wallis test for nonparametric single factor analysis of variance and the Newman-Keuis nonparametric analogue for multiple comparisons, as described by Zar, J. H., *Biostatistical Analysis*. (Prentice-Hall, Inc., Englewood Cliffs, NJ 1984). Independent analyses were carried out for group values on days 7, 14, and 21.

The results are shown in Figure 1. Tumor angiogenesis was significantly inhibited ( $P < 0.05$ ) by the controlled release of minocycline, cortisone alone, and the combination of heparin and cortisone at 7, 14, and 21 days after implantation. The degrees of

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inhibition obtained with the three agents were comparable; there were no statistically significant differences among the three inhibitors at any time. Minocycline decreased tumor-induced angiogenesis by a factor of 4.5, 4.4, and 2.9 at 7, 14, and 21 days, respectively. At the end of the experiment, whereas the corneas with empty polymers had large, invasive, exophytic tumors, none of the corneas with minocycline had such vascular masses, as determined by the histological examination. When implanted alone in the cornea, the polymers containing minocycline, cortisone, and the combination of heparin and cortisone did not induce angiogenesis. Polymers containing heparin alone, however, were noted to promote a mild angiogenic response in the cornea.

Histological examination of the corneas with tumor and minocycline-EVAc polymers confirmed the presence of viable tumor adjacent to the polymer, surviving in an avascular state by day 21. Sections of rabbit cornea, prepared and stained with hematoxylin/eosin, showed the extent of tumor growth and neovascularization 21 days after implantation. Examination shows extensive tumor growth and vascularization in the presence of an empty polymer, and minimal tumor burden and vascularization in the presence of minocycline, while the tumor cells adjacent to minocycline-impregnated polymer are viable, although avascular. Thus, despite the prevention of neovascularization through the cornea, the minocycline does not appear to be directly toxic to the tumor cells.

The results confirmed that minocycline, a semisynthetic tetracycline with anticollagenase activity, was an inhibitor of tumor angiogenesis in the rabbit cornea. Minocycline inhibited angiogenesis to an extent comparable to that of the combination of heparin and cortisone. Histological evidence of viable tumor adjacent to the minocycline-EVAc matrix at 21 days suggests that minocycline itself is not directly cytotoxic to mammalian cells. Other data shows that *in*

-16-

*vitro* minocycline significantly prolongs the doubling time of bovine retinal endothelial cells but not C6 glioma, F98 glioma, or 9L gliosarcoma tumor lines.

**Example 2: Demonstration of specificity of minocycline in inhibiting endothelial cells.**

To test for specificity for inhibiting blood vessel cells, a variety of cells were tested to determine relative sensitivities. One day later increasing doses of minocycline were added, and on the fourth day the cells were counted.

Selective growth inhibition is shown in Figure 2A where pericytes, astrocytes, and endothelial cells were plated. Only endothelial cell growth was inhibited.

The selective inhibition of DNA synthesis, based on <sup>3</sup>H-thymidine uptake, was shown for endothelial cells rather than C6 glioma (brain tumor) cells. The cells were initially plated, and then when nearly confluent, the minocycline was added. Twelve hours later, <sup>3</sup>H-thymidine was added and six hours later, a TCA precipitation was carried out and the CPM were measured.

The results shown in Figure 2B demonstrate that the minocycline specifically inhibited DNA synthesis of endothelial cells rather than C6 glioma tumor cells.

The minocycline was then tested for effectiveness in inhibiting a variety of endothelial cells. The doubling time of the endothelial cells was measured in the presence of the minocycline.

The results shown in Figure 2C show that both paraaortic endothelial cell growth and bovine retinal endothelial cell growth were inhibited by minocycline.

**Example 3: Inhibition of Endothelial Cells using a combination of  $\beta$ -cyclodextrin with minocycline.**

As shown by Figure 3A and 3B, minocycline administered alone in a concentration between 1.0  $\mu$ M and 10  $\mu$ M (Figure 3A) or

-17-

between 5  $\mu$ M and 40  $\mu$ M (Figure 3B) inhibits endothelial cell proliferation by 10 to 20%.  $\beta$ -cyclodextrin inhibits proliferation by 5 to 10% for the lower concentrations and 35 to 60% for the higher concentrations. The combination of  $\beta$ -cyclodextrin with minocycline,  
5 in a ratio of between 0.5 to 1.0 and 2 to 1, inhibits proliferation by between 30 and 40% for the lower concentrations tetracycline and 50 to 60% for the higher concentrations, significantly more than expected from the results observed with each drug administered alone.

Modifications and variations of the compositions of the  
10 present invention, and methods for use, will be obvious to those skilled in the art from the foregoing detailed description. Such modifications and variations are intended to fall within the scope of the appended claims.

SUBSTITUTE SHEET

-18-

We claim.

1. A composition inhibiting angiogenesis comprising an effective amount of a tetracycline or chemically modified tetracycline inhibiting collagenase in a pharmaceutical carrier for administration to a patient having a disorder characterized by uncontrolled proliferation of endothelial cells.
2. The composition of claim 1 wherein the tetracycline is selected from the group consisting of minocycline, chlortetracycline, demeclocycline, doxycycline, lymecycline, methacycline, oxytetracycline, rolitetracycline, tetracycline, 4-de-dimethylaminotetracycline, tetracyclonitrile, 6-deoxy 6-demethyl 4-de-dimethylaminotetracycline, and 7-chloro 4-de-dimethylaminotetracycline.
3. The composition of claim 1 in combination with a chemotherapeutic agent wherein the chemotherapeutic is selected from the group consisting of carmustine (BCNU), 5-fluorouracil,  $\beta$ -cyclodextrin, vincristin, taxol, vinblastin, chlorambucil, cytoxan, alkeran, busulfan, methotrexate, mercaptopurine, bleomycin, adriamycin, thioguanine, chlorotrianisene, cyclophosphamide, 4-HC, and immunotoxins.
4. The composition of claim 1 wherein the effective dosage is between approximately one and 500 micromolar of the tetracycline.
5. The composition of claim 1 wherein the tetracycline is in an ophthalmically acceptable carrier for topical application to the eye.
6. The composition of claim 1 wherein the tetracycline is in a pharmaceutically acceptable carrier for topical application to the skin.
7. The composition of claim 1 wherein the tetracycline is encapsulated in a biocompatible polymeric delivery device.

SUBSTITUTE SHEET

-19-

8. The composition of claim 1 wherein the tetracycline is in a pharmaceutical carrier for internal topical application to ulcers or hemorrhoids.

9. A method for inhibiting angiogenesis and endothelial cell proliferation comprising

administering an effective amount of a tetracycline or chemically modified tetracycline inhibiting collagenase in a pharmaceutical carrier to a patient having a disorder characterized by uncontrolled proliferation of endothelial cells.

10. The method of claim 9 wherein the tetracycline derivative is selected from the group consisting of minocycline, chlortetracycline, demeclocycline, doxycycline, lymecycline, methacycline, oxytetracycline, rolitetracycline, tetracycline, 4-de-dimethylaminotetracycline, tetracyclinonitrile, 6-deoxy 6-demethyl 4-de-dimethylaminotetracycline, and 7-chloro 4-de-dimethylaminotetracycline.

11. The method of claim 9 further comprising administering the tetracycline in combination with a chemotherapeutic agent.

12. The method of claim 11 wherein the chemotherapeutic agent is radiation.

13. The method of claim 11 wherein the chemotherapeutic agent is selected from the group consisting of carmustine (BCNU), 5-fluorouracil,  $\beta$ -cyclodextrin, vincristin, taxol, vinblastin, chlorambucil, cytoxan, alcoran, busulfan, methotrexate, mercaptopurine, bleomycin, adriamycin, thioguanine, chlorotrianisene, cyclophosphamide, 4-HC, and immunotoxins.

14. The method of claim 9 wherein the tetracycline is administered to a concentration of between one and 500 micromolar.

-20-

15. The method of claim 9 further comprising administering the tetracycline in a pharmaceutical vehicle suitable for topical application to the skin.

16. The method of claim 9 further comprising providing the antibiotic in a biocompatible polymeric delivery device.

17. The method of claim 9 further comprising providing the tetracycline in a pharmaceutical vehicle suitable for injection or infusion.

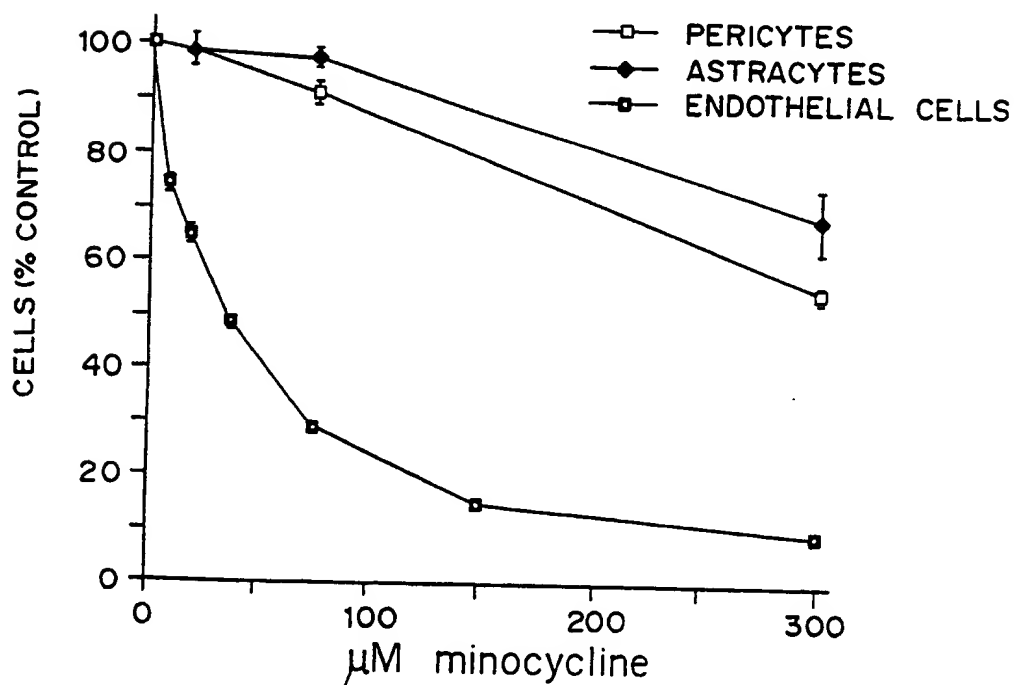
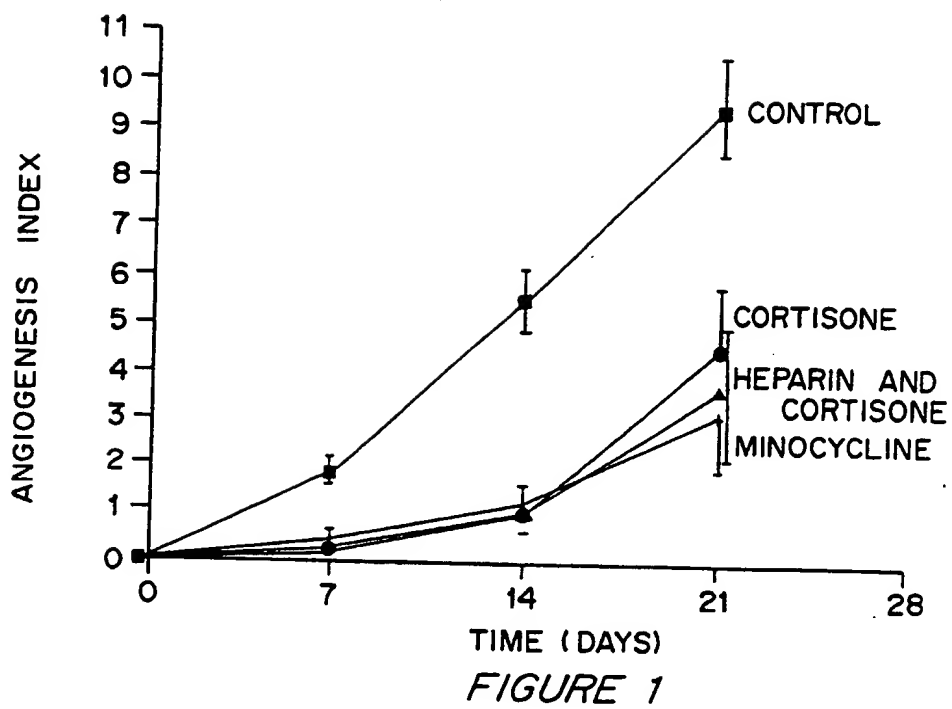
18. The method of claim 9 further comprising administering to the eye of a patient the tetracycline in a pharmaceutical vehicle suitable for topical application to the eye.

19. The method of claim 9 wherein the tetracycline is administered systemically.

20. The method of claim 9 wherein the effective dose is a dose effective in diminishing the number of blood vessels growing into a tumor.

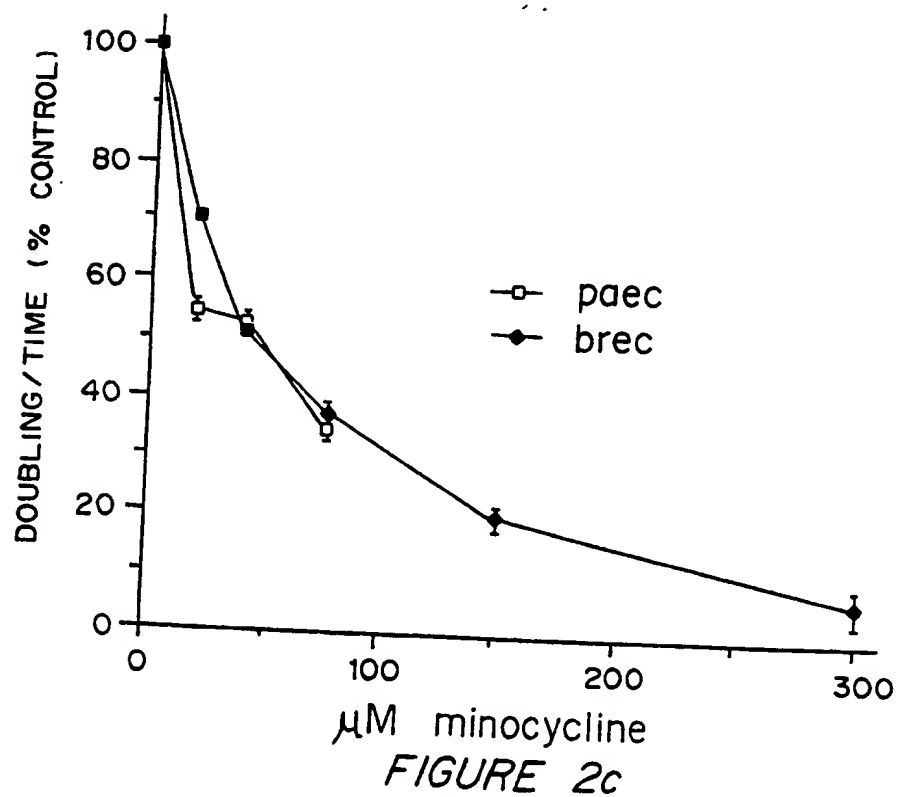
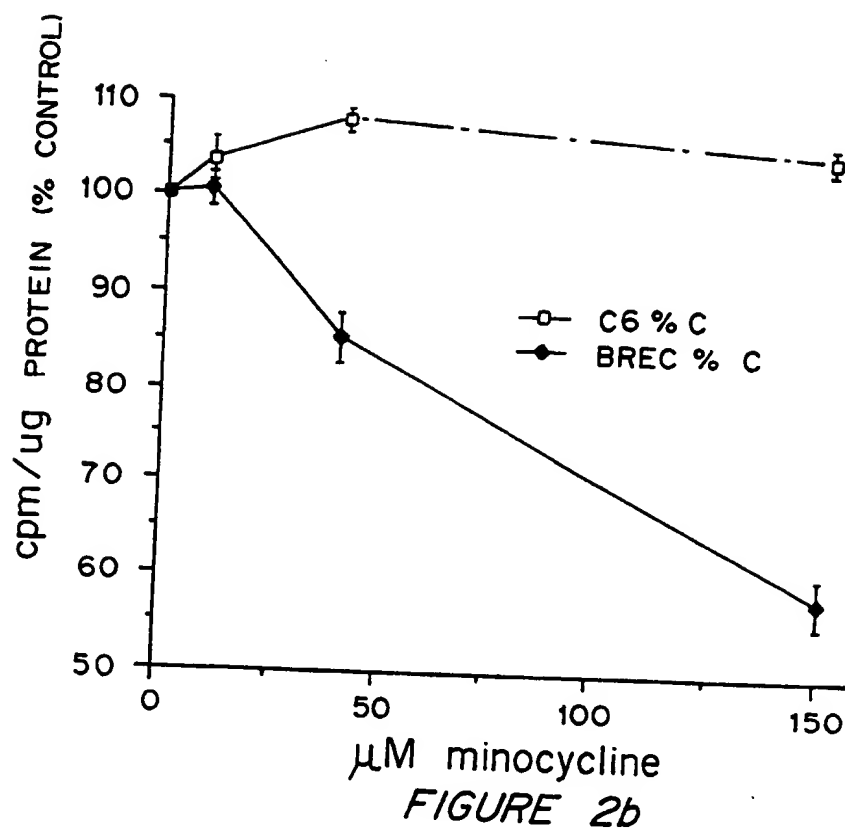
21. The method of claim 9 wherein the effective dose is a dose effective in diminishing the symptoms of eye diseases characterized by abnormal neovascularization.

1/3



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2/3



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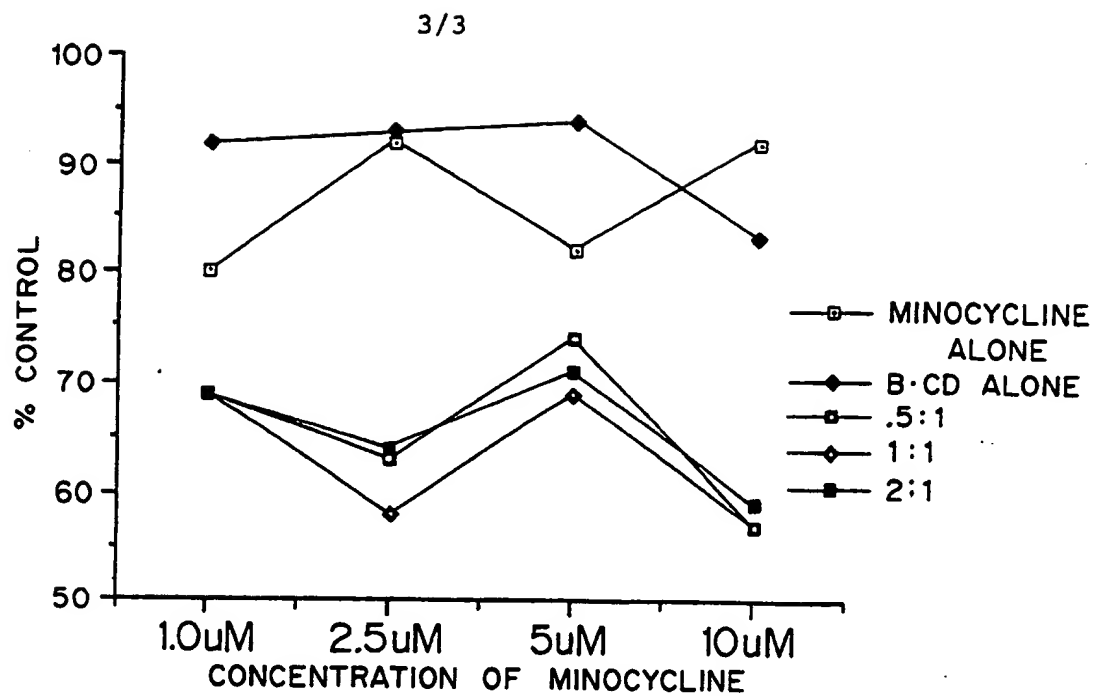


FIG. 3a

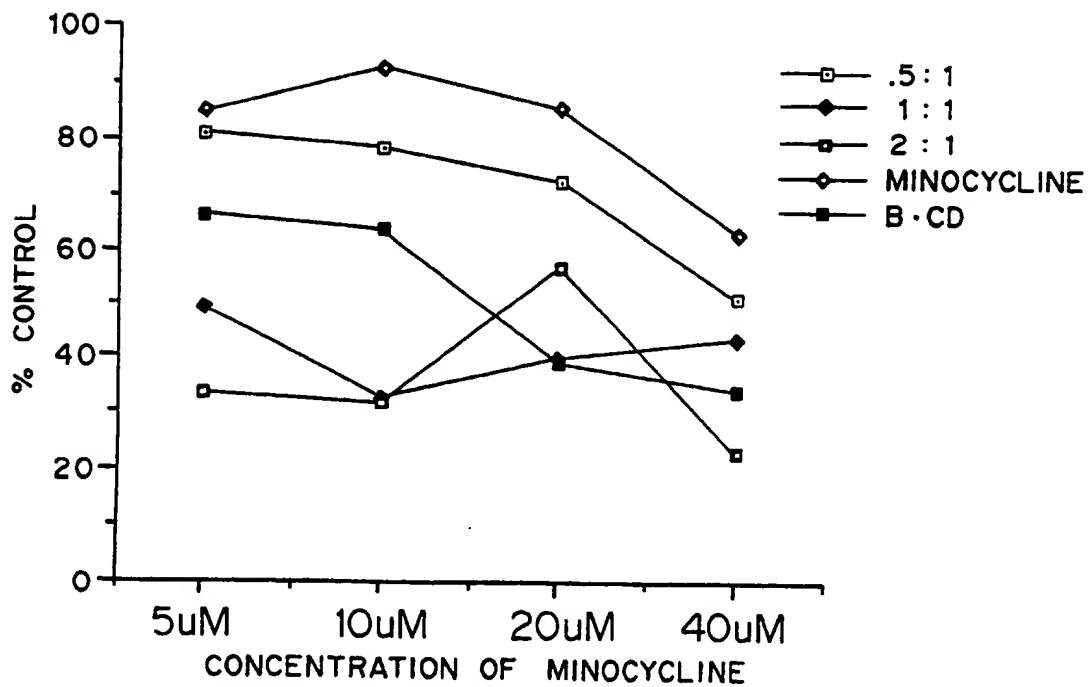


FIG. 3b

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